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Expression of Caveolin-1 Enhances Cholesterol Efflux in Hepatic Cells*

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HepG2 cells were stably transfected with human caveolin-1 (HepG2/cav cells). Transfection resulted in expression of caveolin-1 mRNA, a high abundance of caveolin-1 protein, and the formation of caveolae on the plasma membrane. Cholesterol efflux from HepG2/cav cells was 280 and 45% higher than that from parent HepG2 cells when human plasma and human apoA-I, respectively, were used as acceptors. The difference in efflux was eliminated by treatment of cells with progesterone. There was no difference in cholesterol efflux to cyclodextrin. Cholesterol efflux from plasma membrane vesicles was similar for the two cell types. Transfection led to a 40% increase in the amount of plasma membrane cholesterol in cholesterol-rich domains (caveolae and/or rafts) and a 67% increase in the rate of cholesterol trafficking from intracellular compartments to these domains. Cholesterol biosynthesis in HepG2/cav cells was increased by 2-fold, and cholesterol esterification was reduced by 50% compared with parent HepG2 cells. The proliferation rate of transfected cells was significantly lower than that of non-transfected cells. Transfection did not affect expression of ABCA1 or the abundance of ABCA1 protein, but decreased secretion of apoA-I. We conclude that overexpression of caveolin-1 in hepatic cells stimulates cholesterol efflux by enhancing transfer of cholesterol to cholesterol-rich domains in the plasma membrane.

Cholesterol efflux is the first step in the reverse cholesterol transport (RCT)[1] pathway, removing excess cholesterol from tissues, including the arterial wall, thus preventing the development of atherosclerosis (for review, see Ref. 1). Enhancing cholesterol efflux from the arterial wall may potentially prevent and even reverse intracellular accumulation of cholesteryl esters, a hallmark of atherosclerosis. Enhancing cholesterol efflux from liver cells, the main source of high density lipoprotein (HDL), raises plasma HDL concentrations (2) and consequently increases protection against atherosclerosis by RCT and other mechanisms (3). Enhanced cholesterol efflux can be achieved either by improving the ability of extracellular acceptors to take up cholesterol released from cells or by stimulating cells to release more cholesterol to plasma acceptors. A number of approaches have been tested to improve the ability of cells to release cholesterol. One of these approaches is overexpression of genes involved in the cholesterol efflux pathways. Overexpression of ABCA1 (4), scavenger receptor class B, type I (SR-BI) (5), or sterol 27-hydroxylase (CYP27A1) (6) enhances cholesterol efflux *in vitro*. *In vivo* overexpression of the genes for ABCA1 (7) and SR-BI (8) leads to increased protection against atherosclerosis. Stimulation of cholesterol efflux *in vitro* and enhanced protection against atherosclerosis *in vivo* are also achieved by overexpression of several genes involved in lipoprotein metabolism in plasma, but not normally expressed in most extrahepatic cells. These included apoA-I (9, 10) hormone-sensitive lipase (11), and cholesteryl ester transfer protein (12).

Caveolin-1 is intimately involved in intracellular cholesterol metabolism pathways (for review, see Ref. 13). It has been suggested that caveolin is involved in intracellular cholesterol trafficking (14) and that caveolae are the preferential source of cholesterol for efflux (15, 16). As an essential component of caveolae, caveolin is also involved in numerous signaling pathways (17). Involvement of caveolin in cholesterol trafficking makes it a potential target in the search for approaches to improve cholesterol efflux. There are contradictory reports on the relevance of the level of caveolin expression to cholesterol efflux (18–21), with the overall effect most likely to be cell type-specific. In this work, we demonstrate that overexpression of caveolin-1 in HepG2 cells results in the formation of caveolae and enhancement of cholesterol efflux.

MATERIALS AND METHODS

Cells—HepG2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum, 100 μg/ml penicillin/streptomycin, and 2 mM glutamine (all reagents from JRH Biosciences). Cells were seeded at a density of 1×10^5 cells/well in a collagen-coated 12-well tissue culture plate and cultured for 24 h to 60–80% confluence.

To obtain stable transfectants, HepG2 cells were transfected with the pIRES2-EGFP/caveolin-1 plasmid (a gift of Dr. C. Fielding) using LipofectAMINE Plus reagent (Invitrogen) in serum-free medium for 5 h at 37 °C according to the manufacturer's recommendations. The transfection medium was removed, and fresh complete growth medium was added. Twenty-four hours post-transfection, the cells in one well were split into 10-cm dishes in medium containing 500 μg/ml G418, and the medium was changed every 3–4 days until G418-resistant colonies were clearly evident. Individual colonies were picked into 24-well plates to continue incubation with G418 selection medium. Individual colonies were evaluated for caveolin expression, and a monoclonal line was used for all experiments.

Electron Microscopy—Cells were processed for embedding in resin

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[1] The abbreviations used are: RCT, reverse cholesterol transport; HDL, high density lipoprotein; ABCA1, ATP-binding cassette transporter A1; SR-BI, scavenger receptor class B, type I; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered solution; BSA, bovine serum albumin; RT, reverse transcription.

and sectioned for electron microscopy exactly as described previously (22).

Confocal Microscopy—Cells were grown on sterile collagen-coated glass coverslips to ~60% confluence and fixed in acetone for 20 min at −20 °C. Cells were then washed with phosphate-buffered solution (PBS) and incubated for 1 h with anti-caveolin-1 antibody (Transduction Laboratories, Lexington, KY). Cells were washed again and incubated in the dark for 30 min with Texas Red-labeled anti-rabbit IgG. After mounting onto glass slides, cells were observed under a Zeiss META confocal microscope. Three-dimensional images were reconstructed from images obtained by serial sectioning.

Cholesterol Acceptors—Blood from healthy normolipidemic volunteers (plasma total cholesterol values ranging from 3.4 to 5.0 mmol/liter) was collected in saline containing streptokinase (final concentration of 150 units/ml; Sigma), and plasma was isolated by centrifugation at 1500 × *g* for 15 min at 4 °C. Plasma samples were not pooled, but rather used individually. ApoA-I was isolated as described previously (23).

Cholesterol Efflux—Cells were grown to 80% confluence prior to experiments; the cultures were 100% confluent by the time of incubation with cholesterol acceptors. To label cellular cholesterol, cultures were incubated in serum-containing medium with [1α,2α-^{3}H]cholesterol (specific radioactivity of 1.81 TBq/mmol, final radioactivity of 74 kBq/ml; Amersham Biosciences) for 48 h in a CO_2 incubator. After labeling, cells were washed six times with PBS and incubated for 18 h in serum-free medium. For metabolic labeling of cellular cholesterol, cells were incubated in serum-free Leibovitz L-15 medium with [^{3}H]acetate (specific radioactivity of 95.2 GBq/mmol, final radioactivity of 7.4 MBq/ml; ICN) for 3 h at 15 °C. Cells were washed and incubated for 2 h at 37 °C in serum-free medium containing lipid-free apoA-I (final concentration of 50 μg/ml), cyclodextrin (final concentration of 200 μg/ml), or the indicated concentrations of human plasma. When indicated, a bovine serum albumin (BSA)-progesterone complex (0.5 mg/ml BSA and 10 μg/ml progesterone) was added to the incubation medium, and the same amount of BSA was added to the control incubations. The medium was then collected and centrifuged at 15,000 × *g* for 15 min at 4 °C to remove cellular debris, and the supernatant was counted or used for further analysis. Cells were harvested using a cell scraper and dispensed in 0.5 ml of distilled water, and aliquots were counted or used for further analysis. Metabolically labeled cholesterol was isolated from aliquots of the cells and medium by TLC as described previously (24, 25). Cholesterol efflux is expressed as the percentage of labeled cholesterol transferred from cells to the medium.

Plasma Membrane Vesicle Preparations and Cholesterol Efflux Therefrom—Plasma membrane vesicles were prepared by the method originally described by Bellini *et al.* (26) with modifications introduced by Gaus *et al.* (27). In brief, plasma membrane "blebbing" was induced by incubating cells (labeled with [^{3}H]cholesterol as described above) with 50 mM formaldehyde and 2 mM dithiothreitol (Sigma). The buffer was collected; loose cells were removed by centrifugation; and vesicles were repeatedly centrifuged at 50,000 rpm for 3 h in a Ti-70 rotor to remove formaldehyde and dithiothreitol. Small vesicles were then removed by concentrating the solution using 0.1-μm filters. The plasma membrane vesicles (20 μg of protein) were incubated in 1 ml of DMEM with or without 5% human plasma for 2 h at 37 °C. The mixture was then centrifuged through a 0.1-μm filter at 400 × *g* for 30 s and rapidly washed with DMEM. Aliquots of eluted and retained fractions were counted. The "yield" of separation (*y*; *i.e.* amount of plasma protein eluted through the filter) was determined in a control sample, and cholesterol efflux was calculated as follows: (eluted [^{3}H]cholesterol/total [^{3}H]cholesterol) × 1/*y*.

Cholesterol Trafficking—To label the entire cellular cholesterol pool, cells were incubated in serum-containing medium with [^{14}C]cholesterol (final radioactivity of 0.2 MBq/ml) for 48 h at 37 °C in a CO_2 incubator. After washing, cells were further incubated in serum-free medium for 18 h at 37 °C in a CO_2 incubator. Cells were cooled on ice; [^{3}H]acetate (final radioactivity of 7.4 MBq/ml) was added; and cells were further incubated in Leibovitz L-15 medium for 3 h at 15 °C. Under these conditions, intracellular cholesterol trafficking is blocked, whereas cholesterol biosynthesis proceeds (28, 29). At the end of the incubation, cells were quickly warmed and incubated for 20 min at 37 °C to allow a portion of the newly synthesized cholesterol to be transferred to the plasma membrane. The cells were cooled on ice and washed three times with the ice-cold PBS. Cholesterol oxidase (Roche Applied Science) was added to the cells at a final concentration of 1 unit/ml, and flasks were incubated for 3 h at 4 °C. Under these conditions, only cholesterol in plasma membrane cholesterol-rich domains is oxidized (15, 30), forming cholestenone (referred to as oxysterol throughout), and the reaction is carried out to completion (15). Lipids were extracted from cells and analyzed by TLC as described previously (31). "No-oxidase" and "no-warm-up" controls were included in each experiment. The amount of oxidizable cholesterol was calculated as the amount of [^{14}C]oxysterol or [^{3}H]oxysterol as a fraction of the total non-oxidized [^{3}H]cholesterol in the sample (entire cholesterol pool), thus correcting for losses during lipid separation. The no-oxidase control, which was 10% of the "oxidized" samples, was subtracted from the experimental values.

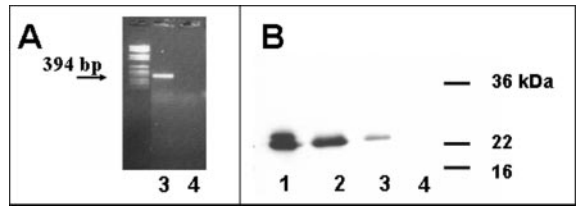

FIG. 1. **Expression of caveolin-1 mRNA (*A*) and protein (*B*) in HepG2 and HepG2/cav cells.** *A*, expression of caveolin-1 mRNA was analyzed by RT-PCR as described under "Materials and Methods." The expected size of the amplicon is 394 bp. *B*, shown is a Western blot of transfected and non-transfected cells using anti-caveolin-1 antibody. *Lane 1*, endothelial cells; *lanes 2* and *3*, different amounts of HepG2/cav cells; *lane 4*, HepG2 cells (same amount of protein as in *lane 3*).

Cholesterol Biosynthesis and Esterification—To assess cholesterol biosynthesis, cells were incubated in serum-free medium with [^{3}H]acetate (final radioactivity of 7.4 MBq/ml) for 20 min at 37 °C in a CO_2 incubator. To assess cholesterol esterification, cells were incubated for 2 h at 37 °C with [^{14}C]oleic acid (specific activity of 2.22 GBq/mmol, final radioactivity of 0.185 MBq/ml; Amersham Biosciences) complexed with BSA (essentially fatty acid-free; Sigma). Cells were washed, and lipids were extracted and analyzed by TLC as described previously (24). Spots of cholesterol and cholesteryl oleate were identified by standards (Sigma), scraped, and counted in a β-counter.

Reverse Transcription (RT)-PCR—Total RNA was extracted from cells following a modification of the guanidinium thiocyanate method (32). The RNA concentration was determined by measuring the absorption at 260 nm. Reverse transcription was carried out in 20 μl containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 5 mM $MgCl_2$, 10 mM dithiothreitol, 40 units of RNase inhibitor, and 50 units of Superscript II reverse transcriptase (Invitrogen) as described previously (6). PCR was performed in a total volume of 50 μl containing 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dNTPS, 100 ng of the appropriate primers (caveolin 5′-primer, GAGGGACATCTCTACACCGTTC; and caveolin 3′-primer, ACTGAATCTCAA TCAGGAAGCTCT), 2 μl of reverse-transcribed cDNA, and 1 units of *Taq* polymerase (PerkinElmer Life Sciences). The reaction was amplified with a DNA thermal cycler (PerkinElmer Life Sciences) for 35 cycles. The amplification profile involved denaturation at 94 °C for 30 s, primer annealing at 58 °C for 30 s, and elongation at 72 °C for 1 min. 10 μl of each PCR reaction was mixed with 2 μl of 6-fold concentrated loading buffer and loaded onto a 1.2% agarose gel containing ethidium bromide. Electrophoresis was carried out at a constant voltage of 100 V for 30 min. The sequences of the fragments amplified by PCR were confirmed by DNA sequencing.

The amount of ABCA1 mRNA in HepG2 and HepG2/cav cells was quantified by real-time RT-PCR according to Su *et al.* (33). Amplification of cDNA was from 50 ng of total RNA. The primers used were as follows: forward, 5′-TCCTCTCCCAGAGCAAAAAGC-3′; and reverse, 5′-GTCCTTGGCAAAGTTCACAAATACT-3′. The probe used was 5′-ACTCCACATAGAAGACTACT-3′, labeled with carboxyfluorescein and carboxytetramethylrhodamine at the 5′- and 3′-ends, respectively. 18 S rRNA was used as an internal control with the probe labeled with VIC™ (Applied Biosystems, Foster City, CA) and carboxytetramethylrhodamine at the 5′- and 3′-ends, respectively. The results are expressed as ΔCt (difference between *Ct* values for ABCA1 and 18 S RNAs). The interassay variation was <2% as determined on 3 separate days. The efficiency of the control and ABCA1 probes was similar.

ApoA-I Synthesis and Secretion—HepG2 or HepG2/cav cells were incubated for 2 h in serum-free medium, and the medium was collected. Cells were scraped, suspended in 500 μl of PBS containing leupeptin and pepstatin (final concentration of 1 μg/ml), and sonicated for 10 s (50% duty). The suspension was centrifuged for 5 min at 15,000 × *g*, and the supernatant was taken for analysis. ApoA-I concentration in the cell lysate and medium was determined by competitive enzyme-linked immunosorbent assay as described previously (34).

Western Blotting—Cells were lysed in radioimmune precipitation assay

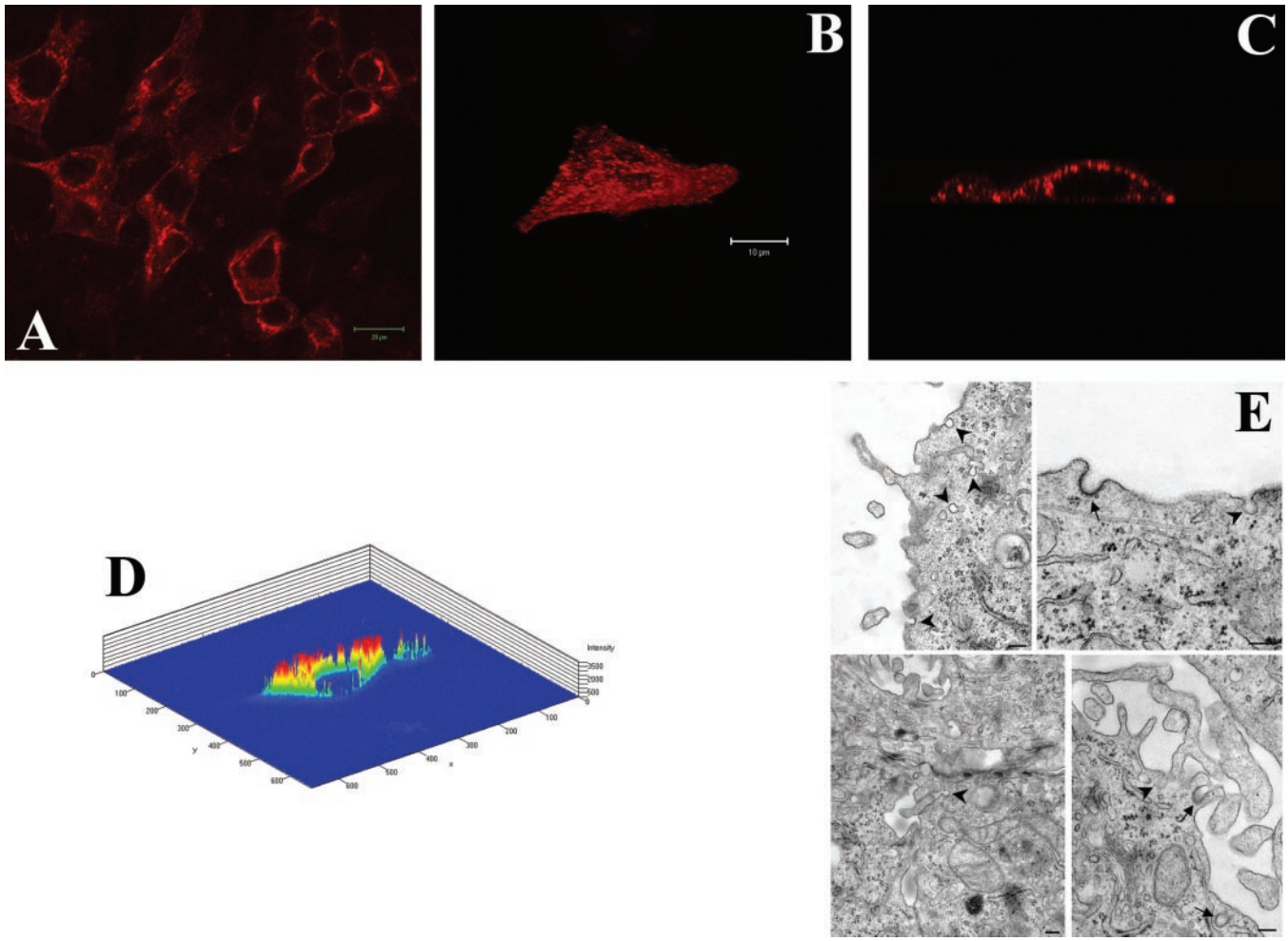

FIG. 2. **Confocal and electron microscopy of caveolae in HepG2/cav cells.** *A*, confocal microscopy. Cells were fixed in acetone for 20 min, washed with PBS, incubated with anti-caveolin-1 antibody, washed again, and incubated in the dark with Texas Red-labeled anti-rabbit IgG. *Bar* = 20 μm. *B*, three-dimensional confocal image of a HepG2/cav cell processed as described for *A* and reconstructed from serial sectioning. *Bar* = 10 μm. *C*, cross-section of the image in *C*. *D*, intensity plot of the image in *C*. *Blue* and *yellow* correspond to a low and medium abundance, respectively, of caveolin in a particular section, and *red* corresponds to a high abundance of caveolin. *E*, electron microscopy. Cells were processed for embedding in Epon, and sections were cut parallel to the substratum. Uncoated flask-shaped pits or vesicular profiles typical of caveolae are evident in the transfected cells (indicated by *arrowheads*). These contrast with larger clathrin-coated pits (indicated by *arrows*). *Bars* = 100 nm.

buffer, and proteins were separated on a 6% (ABCA1) or 15% (caveolin) SDS-polyacrylamide gel, followed by immunoblotting using either rabbit anti-ABCA1 serum (raised against a recombinant fragment of human ABCA1 (amino acids 1314–1450)) or rabbit anti-human caveolin-1 antibody (Transduction Laboratories). Bands were visualized by chemiluminescence development and quantified by densitometry.

Statistical Analysis—All experiments were reproduced two to four times, and representative experiments are shown. Unless otherwise indicated, experiments were performed in quadruplicate. Means ± S.D. are presented. Student's *t* test was used to determine statistical significance of the differences.

RESULTS

Stable Transfection of HepG2 Cells with Caveolin-1—When HepG2 cells were stably transfected with caveolin-1 (HepG2/cav cells), a strong signal of 394 bp corresponding to caveolin mRNA was detected by RT-PCR (Fig. 1*A*, *lane 3*). Expression of caveolin was also analyzed by Western blot analysis using specific anti-caveolin-1 antibody. A single band of 22 kDa migrating at the same position as caveolin from endothelial cells was detected in HepG2/cav cells (Fig. 1*B*, *lanes 1–3*). No expression of caveolin-1 mRNA or caveolin protein was detected in non-transfected HepG2 cells (Fig. 1, *A* and *B*, *lane 4*). This confirms the previous findings of Fujimoto *et al.* (35) that HepG2 cells do not normally express caveolin. The plasmid used for transfection contained green fluorescent protein, enabling evaluation of the efficiency of transfection. After the final cloning, ~90% of the cells expressed green fluorescent protein.

Cells were also treated with anti-caveolin-1 antibody and studied using confocal microscopy. HepG2/cav cells stained with anti-caveolin antibody (Fig. 2*A*), whereas control HepG2 cells showed no staining (data not shown). Caveolin in HepG2/cav cells was mostly present on the plasma membrane; however, significant staining was also found in the perinuclear area. This is consistent with the caveolin present in plasma membrane caveolae and the Golgi and is similar to the pattern observed in caveola-rich cholesterol-loaded endothelial cells (36), smooth muscle cells (37), and mouse peritoneal macrophages (38). The pattern of distribution of caveolin between different regions in the plasma membrane of HepG2/cav cells was studied by reconstructing three-dimensional confocal images. Most caveolin was found on the dorsal side of cells, as evident from three-dimensional (Fig. 2*B*) and cross-sectional (Fig. 2*C*) images as well as well as from quantitative analysis of the intensity distribution of caveolin-1 staining in transfected cells (Fig. 2*D*). Weak staining was found on the ventral parts of the membrane (Fig. 2, *B* and *C*).

The caveolin-1-transfected cells were also examined by electron microscopy. Surface-connected pits with the typical morphology of caveolae and ~65 nm in size were observed in the

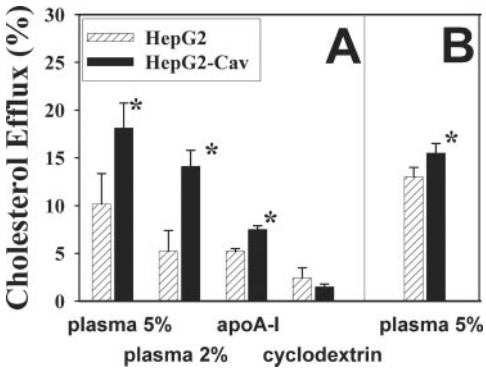

FIG. 3. **Cholesterol efflux to human plasma and lipid-free apoA-I.** Transfected and non-transfected cells were labeled with [^{3}H]cholesterol (*A*) or [^{3}H]acetate (*B*) as described under "Materials and Methods." Cells were then incubated with the indicated concentrations of human plasma, human lipid-free apoA-I (final concentration of 50 μg/ml), cyclodextrin (final concentration of 200 μg/ml), or serum-free medium alone for 2 h at 37 °C in a CO_2 incubator. *A*, the medium was collected; cells were washed; and the amount of radioactivity in the cells and medium was determined by liquid scintillation spectrometry. *B*, cholesterol was isolated from the cells and medium and separated by TLC as described under "Materials and Methods." Cholesterol efflux is expressed as the percentage of labeled cholesterol that moved from cells to the medium (*i.e.* radioactivity in the medium/radioactivity in the medium + radioactivity in the cells). Means ± S.D. of quadruplicate determinations are shown. *, $p < 0.01$ *versus* non-transfected cells.

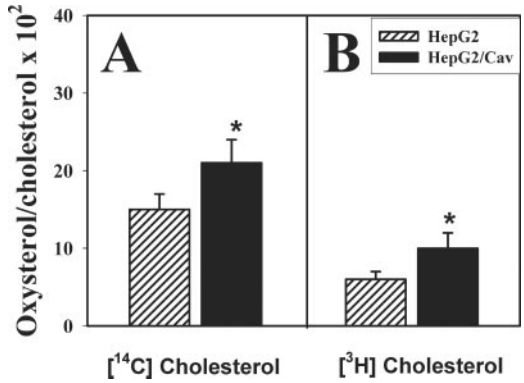

FIG. 4. **Evaluation of the amount of cholesterol in cholesterol-rich domains (*A*) and the rate of cholesterol trafficking from the endoplasmic reticulum to cholesterol-rich domains (*B*).** The entire cellular cholesterol pool was labeled by incubation in serum-containing medium with [^{14}C]cholesterol for 48 h at 37 °C in a CO_2 incubator. After washing, cells were further incubated in serum-free medium for 18 h at 37 °C in a CO_2 incubator. Cells were cooled on ice; [^{3}H]acetate was added; and cells were further incubated for 3 h at 15 °C. At the end of the incubation, cells were quickly warmed and incubated for 20 min at 37 °C to allow a portion of the newly synthesized cholesterol to be transferred to the plasma membrane. The cells were cooled on ice and washed three times with ice-cold PBS. Cholesterol oxidase was added to the cells at a final concentration of 1 unit/ml, and flasks were incubated for 3 h at 4 °C. Lipids were extracted from cells and analyzed by TLC as described under "Materials and Methods." The amount of oxidizable cholesterol was calculated as the amount of [^{14}C]oxysterol or [^{3}H]oxysterol as a fraction of the total non-oxidized [^{14}C]cholesterol in the sample (entire cholesterol pool). *, $p < 0.02$.

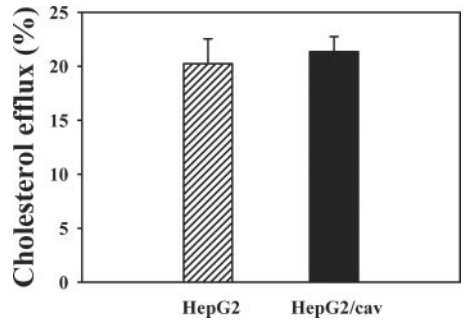

FIG. 5. **Cholesterol efflux from plasma membrane vesicles.** Plasma membrane vesicles were prepared as described under "Materials and Methods." The vesicles (20 μg of protein) were incubated in 1 ml of DMEM with or without 5% human plasma for 2 h at 37 °C. The mixture was then centrifuged through a 0.1-μm filter at 400 × *g* for 30 s and rapidly washed with DMEM. Aliquots of eluted and retained fractions were counted. The yield of separation (*y*; *i.e.* amount of plasma protein eluted through the filter) was determined in a control sample, and cholesterol efflux was calculated as follows: (eluted [^{3}H]cholesterol/total [^{3}H]cholesterol) × 1/*y*. Background values (*e.g.* efflux in the absence of plasma) were subtracted.

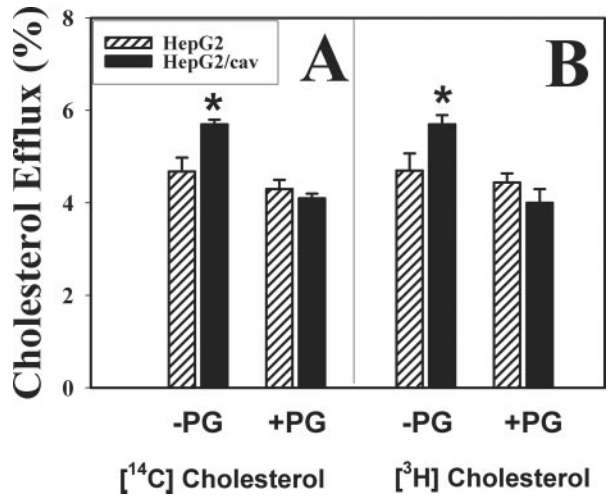

FIG. 6. **Effect of progesterone on cholesterol efflux from HepG2 and HepG2/cav cells.** Transfected and non-transfected cells were labeled with [^{14}C]cholesterol (*A*) or [^{3}H]acetate (*B*) as described under "Materials and Methods." Cells were then incubated with BSA-progesterone (10 μg/ml progesterone (*PG*)) or BSA alone and 5% human plasma or with serum-free medium for 1 h at 37 °C in a CO_2 incubator. *A*, the medium was collected; cells were washed; and the amount of radioactivity in the cells and medium was determined by liquid scintillation spectrometry. *B*, cholesterol was isolated from the cells and medium and separated by TLC as described under "Materials and Methods." Cholesterol efflux is expressed as the percentage of labeled cholesterol that moved from cells to the medium (*i.e.* radioactivity in the medium/radioactivity in the medium + radioactivity in the cells). Means ± S.D. of quadruplicate determinations are shown. *, $p < 0.01$ *versus* non-transfected cells.

transfected cells on both the dorsal surfaces of the cells and in regions of cell-cell contacts, but not in control cells (Fig. 2*E*). However, caveola abundance was low, with, on average, less than one caveolar profile/cell profile.

Cholesterol Efflux—To evaluate the effect of caveolin expression on cholesterol efflux, cellular cholesterol was labeled with [^{3}H]cholesterol, and cells were incubated with human plasma, lipid-free apoA-I, cyclodextrin, and medium alone. Compared with HepG2 cells, the efflux from HepG2/cav cells was 80, 280, and 45% higher ($p < 0.01$ for all) when 5% plasma, 2% plasma, and 50 μg/ml apoA-I, respectively, were used as acceptors (Fig. 3*A*). There was no significant difference in cholesterol efflux from the two cell types to cyclodextrin (200 μg/ml), a nonspecific cholesterol acceptor. When cellular cholesterol was metabolically labeled with [^{3}H]acetate, the efflux from HepG2/cav cells to 5% plasma was 20% higher than that from HepG2 cells ($p < 0.01$) (Fig. 3*B*).

Intracellular Cholesterol Trafficking—Caveolae are considered to be a principal source of cholesterol for efflux (15, 16). It has also been suggested that caveolin is involved in transporting cholesterol from intracellular compartments to the plasma membrane pool (14), where it becomes accessible for efflux. Introduction of caveolin into cells not expressing it naturally and formation of caveolae may also increase the amount of

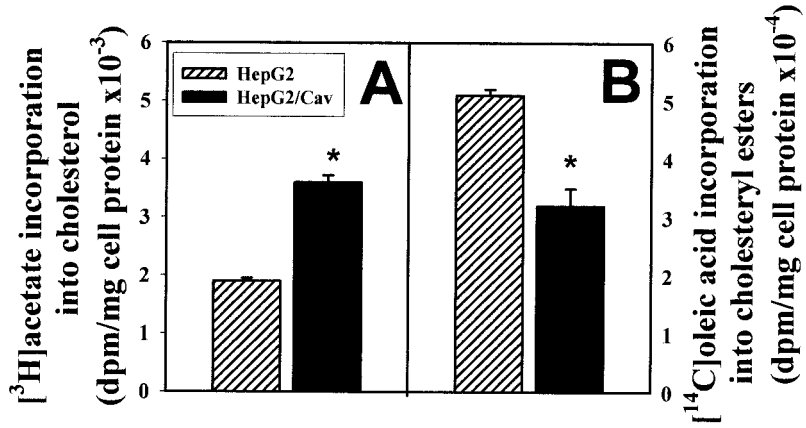

FIG. 7. **Cholesterol biosynthesis (*A*) and esterification (*B*) in HepG2 and HepG2/cav cells.** Cells were incubated in serum-free medium with [^{3}H]acetate for 20 min (*A*) or with [^{14}C]oleic acid for 2 h (*B*) at 37 °C in a CO_2 incubator. Cells were washed. and lipids were extracted and analyzed by TLC as described under "Materials and Methods." *, $p < 0.001$.

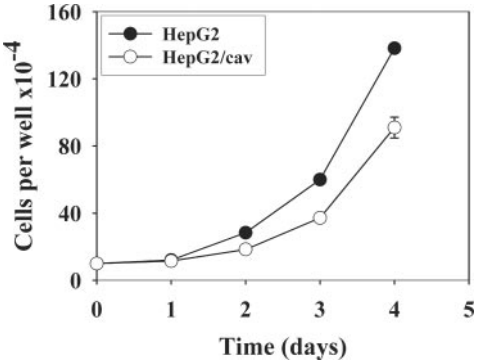

FIG. 8. **Proliferation of HepG2 and HepG2/cav cells.** Cells were seeded at the same density (1×10^5 cells/well) in 6-well plates, and the number of cells was counted every day. ●, HepG2 cells; ○, HepG2/cav cells.

TABLE I
Synthesis and secretion of apoA-I by HepG2 and HepG2/cav cells
The amount of apoA-I in the cells and medium after a 2-h incubation was analyzed by competitive enzyme-linked immunosorbent assay as described under "Materials and Methods."

Cells	Intracellular apoA-I	Secreted apoA-I	
	mg/mg cell protein	*mg/mg cell protein*	*mg/ml*
HepG2	0.25 ± 0.01	2.8 ± 0.2	5.4 ± 0.3
HepG2/cav	0.21 ± 0.12	0.8 ± 0.1[a]	2.4 ± 1.1[b]

[a] $p < 0.001$.
[b] $p < 0.01$.

cholesterol in pools accessible for efflux. To assess these possibilities, cells were labeled with [^{14}C]cholesterol, allowing labeled cholesterol to equilibrate among all cellular pools. Cells were then treated with cholesterol oxidase under conditions in which only cholesterol in cholesterol-rich domains on the cell surface (rafts and/or caveolae) is oxidized. The relative amount of cholesterol in cholesterol-rich domains in HepG2/cav cells was 40% higher compared with that in HepG2 cells ($p < 0.02$) (Fig. 4*A*). To assess the rate of cholesterol trafficking, cells were incubated with [^{3}H]acetate for 3 h at 15 °C. Cells were then warmed up for 20 min at 37 °C to allow newly synthesized cholesterol to move to the plasma membrane before treatment of cells with cholesterol oxidase. The amount of newly synthesized [^{3}H]cholesterol that moved to the cholesterol-rich domains in the plasma membrane was 67% higher in HepG2/cav cells compared with that in HepG2 cells ($p < 0.02$) (Fig. 4*B*).

These experiments indicate that cholesterol efflux in cells overexpressing caveolin-1 is enhanced due to increased transfer of cholesterol to plasma membrane pools accessible for efflux. However, they do not distinguish between contributions of increased cholesterol flow to these domains and higher steady-state concentrations of cholesterol in this pool. To investigate the issue further, we compared the efflux of cholesterol from plasma membrane vesicles prepared from HepG2 and HepG2/cav cells. Cholesterol efflux from the vesicles, which reflects the concentration of cholesterol in the plasma membrane without contribution of cholesterol flow from intracellular compartments, was similar for the two cell types (Fig. 5). This is consistent with a lack of difference between the two cell types in cholesterol efflux to cyclodextrin, which is an indirect measure of plasma membrane cholesterol content (Fig. 3*A*) (39).

Furthermore, we treated cells with progesterone, a potent inhibitor of intracellular cholesterol trafficking (14, 31). To minimize nonspecific equilibration of cholesterol between cellular compartments, the duration of efflux incubation was shortened to 1 h in these experiments, a change in conditions that affected the magnitude of the difference in efflux from the two cell types. Nevertheless, the efflux of [^{14}C]cholesterol from HepG2/cav cells to 5% plasma was significantly higher compared that from parent HepG2 cells; this difference was completely eliminated in the presence of 10 μg/ml progesterone (Fig. 6*A*). Progesterone also eliminated the difference in efflux of intracellular cholesterol after labeling cells metabolically with [^{3}H]acetate (Fig. 6*B*). These experiments indicate that overexpression of caveolin-1 enhances cholesterol efflux mainly by stimulating intracellular cholesterol trafficking, with apparently increased steady-state concentrations of cholesterol in plasma membrane cholesterol-rich domains contributing little to the efflux.

Cholesterol Biosynthesis and Esterification—Cholesterol biosynthesis and esterification in HepG2 and HepG2/cav cells were assessed as an indirect measure of changes in intracellular cholesterol content. The rate of [^{3}H]acetate incorporation into cholesterol in HepG2/cav cells was almost twice that in HepG2 cells ($p < 0.001$) (Fig. 7*A*). In contrast, the rate of [^{14}C]oleic acid incorporation into cholesteryl esters in HepG2/cav cells was 40% lower compared with that in HepG2 cells ($p < 0.001$) (Fig. 7*B*).

Cell Proliferation—Caveolin and caveolae are tightly linked to cell growth by changing cellular cholesterol content (21) and/or through platelet-derived growth factor signaling (37). When the rate of proliferation was analyzed in HepG2 and HepG2/cav cell cultures seeded at the same density, the rate was significantly slower in HepG2/cav cells. 51% fewer cells were found in HepG2/cav cultures than in HepG2 cultures after 4 days of culturing, when the cultures were approaching confluency ($p < 0.001$) (Fig. 8). This finding is consistent with reports of Fielding *et al.* (21), who demonstrated inhibition of human skin fibroblast proliferation after transfection with caveolin-1.

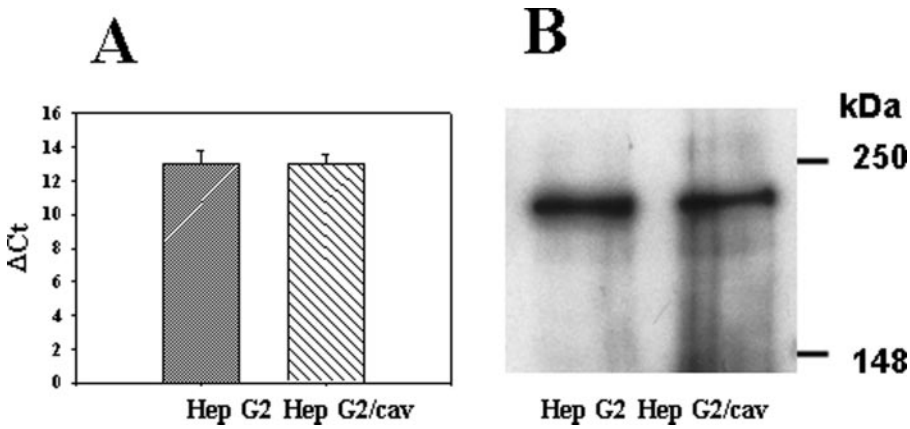

FIG. 9. **ABCA1 expression in HepG2 and HepG2/cav cells.** *A*, abundance of ABCA1 mRNA in two cell types as determined by real-time RT-PCR; *B*, Western blot of HepG2 and HepG2/cav cells using anti-ABCA1 antibody.

Secretion of ApoA-I—As a hepatic cell line, HepG2 cells produce and secrete apoB-containing lipoproteins (mainly low density lipoprotein) and apoA-I (40), which may contribute to the release of cholesterol from cells. We found that HepG2 cells produced substantial amounts of apoA-I and that most of this was secreted into the medium (Table I). However, the amount of apoA-I secreted by both cell types during a 2-h incubation was <11% of the amount of exogenous apoA-I added. Interestingly, the amount of apoA-I synthesized and secreted by HepG2/cav cells was three times lower compared with nontransfected cells (Table I). Thus, the observed stimulation of cholesterol efflux is not related to apoA-I synthesis and its secretion by the cells.

Background efflux (*i.e.* efflux in the absence of any exogenous acceptors) was <10% and was similar for the two cell types. This discounted the possibility that cholesterol released with secreted apoB- and apoA-I-containing lipoproteins contributed significantly to the observed differences in cholesterol efflux.

Expression of ABCA1—It was demonstrated by Orso *et al.* (41) that mutations of ABCA1 affect caveolin-1 expression and processing. In view of a possible link between expression of caveolin and ABCA1, we investigated the effect of stable transfection with caveolin on ABCA1 expression. However, when analyzed by real-time RT-PCR, no difference in the abundance of ABCA1 mRNA between the two cell types was found (Fig. 9*A*). There was also no significant difference in the abundance of ABCA1 protein as measured by Western blotting, during which a single band of 220 kDa was observed (Fig. 9*B*).

DISCUSSION

The major finding of this study is that stable transfection of HepG2 cells with caveolin-1 enhances cholesterol efflux. The effect was independent of lipoprotein secretion and ABCA1 expression. Cholesterol efflux is the first step in the RCT pathway, and the liver is usually considered the destination rather than the origin of RCT. However, it was recently suggested that the liver may be the major source of HDL cholesterol in plasma (7). Thus, the liver may play a dual role in the regulation of RCT by being both the origin and destination of the pathway.

The effect of transfection of cells with caveolin-1 on intracellular cholesterol metabolism and cholesterol efflux was studied previously, but the results were inconsistent. Smart *et al.* (14) demonstrated that transport of newly synthesized cholesterol to plasma membrane caveolae is significantly enhanced after transfection of L1210-JF cells with caveolin. Fielding *et al.* (21) demonstrated that transfection of human fibroblasts with caveolin increases cholesterol efflux. Arakawa *et al.* (42) demonstrated that inhibition of caveolin-1 expression inhibits cholesterol efflux in THP-1 cells. In contrast, Frank *et al.* (20) showed that inhibition of caveolin-1 expression stimulates cholesterol efflux in NIH-3T3 fibroblasts. Matveev *et al.* (43) did not observe changes in cholesterol efflux from J774 or RAW cells after transfection with caveolin. Wang *et al.* (18) and Frank *et al.* (19) also found that transfection of HEK-293T and FRT cells with caveolin has no effect on cholesterol efflux. The likely reason for the differences observed is that the role of caveolin in cholesterol metabolism is cell type-specific. In this study, we did not observe stimulation of cholesterol efflux after transient overexpression of human caveolin-1 in CHOP and RAW 264.7 cells, in which there was at least a 6-fold increase in cellular caveolin content after transfection (data not shown). At the same time, overexpression of SR-BI[2] or CYP27A1 (6) in these cells enhances cholesterol efflux. Another example is that expression of caveolin is down-regulated by HDL in NIH-3T3 fibroblasts (20), but up-regulated by apoA-I in human skin fibroblasts (31). A possible cause for the cell type-specific differences might be the extent of involvement of caveolin and caveolae in cholesterol efflux and/or the extent of formation of caveolae after overexpression of caveolin, both of which may vary widely from one cell type to another. None of the previous studies evaluated the formation of new caveolae or a change in abundance of existing caveolae after transfection of cells with caveolin-1. The presence of caveolin is not always associated with formation of caveolae (44), and we did not find a dramatic increase in the number of caveolae after transfection of CHOP cells with caveolin (data not shown). We have previously demonstrated that caveolin may not only be localized in caveolae, but may also be present in intracellular lipid bodies and the Golgi (45). In this study, we found that a proportion of HepG2/cav cells did not form caveolae, but rather contained caveolin in intracellular compartments. Distribution of caveolin within the cell and formation of caveolae may be critical factors regulating cholesterol efflux. The effect of transfection with caveolin may also depend on the ability of caveolin-1 to form complexes with other proteins involved in intracellular trafficking, such as heat shock protein (46) and caveolin-2 (38). Finally, the results obtained may depend on the exact pathway investigated in a particular study. Frank *et al.* (19) studied SR-BI-dependent cholesterol efflux in cells overexpressing SR-BI with or without the concomitant expression of caveolin. The recent findings that SR-BI may not be co-localized with caveolae (47) and that caveolae are not required for its proper function (44) suggest that the SR-BI-dependent pathway might not depend on caveolae at least in some cell types. Wang *et al.* (18) used very high concentrations of acceptors, which could make the contribution of the caveolin-independent diffusion pathway of cholesterol efflux overwhelming.

The most likely mechanism of enhanced cholesterol efflux after overexpression of caveolin is stimulation of the flow of

[2] D. Sviridov, G. Escher, and Z. Krozowski, unpublished data.

cholesterol to cholesterol-rich domains in the plasma membrane. The following findings support this suggestion. (i) Caveolae were formed as a result of transfection of HepG2 cells with caveolin. (ii) The rate of trafficking of newly synthesized cholesterol to cholesterol-rich domains and its efflux increased in HepG2/cav cells. (iii) The efflux of cholesterol from plasma membrane vesicles, where the contribution of cholesterol trafficking is eliminated, was similar for the two cell types. (iv) Progesterone, an inhibitor of intracellular cholesterol trafficking, eliminated the difference in cholesterol efflux from the two cell types. (v) The concentration of cholesterol in the endoplasmic reticulum decreased, as evidenced by an increase in cholesterol biosynthesis and inhibition of cholesterol esterification. The latter indicates that there is constant removal of cholesterol from intracellular compartments. In addition, we excluded a contribution of two other obvious possibilities of an indirect effect of transfection with caveolin: secretion of *de novo* synthesized lipoproteins and expression of ABCA1. Although there is an apparent increase in the cholesterol content in plasma membrane cholesterol-rich domains, our data suggest that the main mechanism of the effect of caveolin on cholesterol efflux is stimulation of transfer of cholesterol from intracellular compartments to cholesterol-rich domains in the plasma membrane. Cholesterol in these domains can then be released to specific extracellular acceptors if they are available or transferred to other regions in the plasma membrane (14).

In this study, we have demonstrated that transfection of HepG2 cells with caveolin results in an increase in cholesterol efflux. The potential benefit of increasing cholesterol efflux is that this would prevent or slow accumulation of cholesterol in the artery wall, preventing development of atherosclerosis. However, the effect of caveolin appears to be cell type-specific, and our finding may be relevant only to liver cells. Nevertheless, there are several ways in which overexpression of caveolin may be exploited for the treatment of atherosclerosis. Frank *et al.* (48) demonstrated that overexpression of caveolin in the liver leads to an increase in plasma HDL concentration. Although these authors attributed this to inhibition of the selective uptake of HDL cholesteryl esters, an increase in cholesterol efflux may also contribute. Enhancement of cholesterol efflux by overexpression of ABCA1 in the liver also leads to a significant increase in plasma concentrations of HDL (2). Higher plasma HDL levels may result in enhanced protection against atherosclerosis due to either enhanced RCT or other anti-atherogenic properties of HDL. The effects of ABCA1 and caveolin might be additive, providing even greater protection against atherosclerosis. The effect of caveolin transfection on cholesterol efflux from vascular cells might depend on whether or not transfection leads to proper intracellular distribution of caveolin and the formation of complexes with other proteins. It is possible that caveolin-1 might need to be cotransfected with other genes, *e.g.* caveolin-2. Finally, overexpression of caveolin leads to the inhibition of cell proliferation. This finding is consistent with those of Fielding *et al.* (21) and Peterson *et al.* (37) and provides another mechanism that would attenuate the progression of atherosclerosis.

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Expression of Caveolin-1 Enhances Cholesterol Efflux in Hepatic Cells

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